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Role of Motility in Adherence to and Invasion of a Fish Cell Line by *Vibrio anguillarum*

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To understand further the role of the flagellum of *Vibrio anguillarum* in virulence, invasive and adhesive properties of isogenic motility mutants were analyzed by using a chinook salmon embryo cell line. Adhesion was unaffected but invasion of the cell line was significantly decreased in nonmotile or partially motile mutants, and the chemotactic mutant was hyperinvasive. These results suggest that active motility aids invasion by *V. anguillarum*, both in vivo and in vitro.

The polar flagellum of *Vibrio anguillarum* is a complex structure containing four or possibly five flagellin proteins (13, 15) and is covered in a membranous sheath (16). Recent studies have shown that the flagellum aids *V. anguillarum* in crossing the fish integument (15, 18). Once the bacterium has crossed this barrier, the flagellum is no longer required for virulence. In many pathogenic bacteria, the flagellum has been shown to play a role in adherence to or invasion of host tissues or cell line cultures (20). During the initial steps of entry into the host, the flagellum may serve either as a motility organelle that aids the penetration of mucosal tissues or as an organelle that carries an adhesin for binding to the mucosal tissue. Little is known about the adherence and invasion mechanisms of *V. anguillarum*, the causative agent of vibriosis in marine fish. *V. anguillarum* has been shown to adhere to a chinook salmon embryo (CHSE) cell line, 214 (8, 22); to a fish epithelial cell line, epithelioma papulosum cyprini carp cells (23); to intestinal tissue sections from rainbow trout (5); and to mucus-coated glass slides (8). The adherence to the CHSE cells and to the mucus correlated with pellicle formation, suggesting that *V. anguillarum* has an adhesin which is needed for attachment to cells (8). Wang et al. (23) have also shown that some *V. anguillarum* strains can invade epithelioma papulosum cyprini carp cells and that internalization likely requires the involvement of host cell microfilaments and microtubules.

Does some part of the polar flagellum act as an adhesin, or is it active motility that is needed for invasion of the fish host? To answer this question, a flagellar motor gene, *motY*, was cloned and mutated, creating a strain with an intact but paralyzed flagellum. To obtain a probe for cloning *motY*, two degenerate inosine-containing oligonucleotides (5′-GGACTAG TGTIAA(TC)ACICCITTIGA(AG)TG(TC)CA-3′ and 5′-CT CGAGCTCCG(CT)TTICC(AG)TAICC(TC)TGIAC(TC)T G-3′) were designed based on the *Vibrio parahaemolyticus motY* gene (12) and used in PCR as previously described (13), except that the Stratagene PCR optimization kit was used. The PCR fragment obtained was used as a probe to screen a previously described lambda-based *V. anguillarum* chromosomal DNA library (14), using previously described hybridization conditions (15). The *motY* gene was sequenced, and the deduced product is 80% identical to the MotY proteins from *V. parahaemolyticus* (12) and *Vibrio alginolyticus* (17). *V. anguillarum* MotY, like those of *V. parahaemolyticus* (12) and *V. alginolyticus* (17), has a C-terminal domain that is thought to stabilize the protein in the peptidoglycan, which allows MotY to act as a stator for the sodium-driven motor of the polar flagella. The *motY* gene has both possible consensus σ^{54}-like (3) and σ^{28}-like promoter sequences (9). Moreover, the genetic organization of the *motY* locus is similar to that of both *V. parahaemolyticus* (12) and *V. alginolyticus* (17). *motY* maps in a region which is void of other flagellar genes but which contains open reading frames similar to those of the RNase T locus in *Escherichia coli* (6). However, in *V. anguillarum*, open reading frame 2 of *V. parahaemolyticus* (12) was not found.

For functional analyses of the MotY protein, an in-frame deletion of the entire *motY* gene from the region encoding the start methionine to that corresponding to the last coded amino acid was made as described previously (15). The growth rate of the resulting mutant, which can affect both the motility and virulence analyses, was tested and was similar to that of the wild type. To show that the motor and not the structure of the flagellum is affected by the mutation, microscopic analysis and soft-agar assays were done as previously described (13, 15). The *motY* mutant was unable to swim from its point of inoculation in soft agar (Table 1) and was also nonmotile when viewed under the light microscope. When the mutant was analyzed by electron microscopy, the structure of the flagellum was found to be similar to that of the wild type's. These data indicate that the *motY* mutant has an intact but paralyzed flagellum.

To confirm that the loss of motility was due to the *motY* gene product, the wild-type gene was inserted back into the chromosome of the *motY* mutant strain by using the suicide plasmid pNQ705-1 (13) carrying the wild-type gene and its flanking DNA. Soft-agar motility assays showed that 92% of wild-type motility was regained when the mutant gene was complemented with the wild-type gene (Table 1).

When rainbow trout were infected with the *motY* mutant via previously described methods (15), the *motY* mutant showed a 750-fold decrease in virulence from that of the wild type when immersed into bacterium-containing seawater, 3×10^6 versus 4×10^3 bacteria/ml. No loss in virulence was seen when the

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TABLE 1. Adhesion and invasion assays for *V. anguillarum* motility mutants

Strain	Mutation	Intact flagellum	% Motility[a]	% Adherence[b] (mean ± SD)	No. of bacteria/ CHSE cell	% Intracellular bacteria (mean ± SD)[b]
NB10	None (wild type)	+	100	6 ± 3	1.2	28 ± 2
PO2	*motY*	+	0	13 ± 4	1.3	10 ± 1
PO2/pNQMotY	*motY*/pNQMotY	+	92	6 ± 2	1.1	33 ± 5
RO8	*flaA*	Partial flagellum	53	10 ± 2	1.2	14 ± 3
RO8/pFlaA	*flaA*/pFlaA	+	87	8 ± 3	1.2	29 ± 3
OTR7	*flhA*	−	0	14 ± 6	1.4	22 ± 8
OTR27	*cheR*	+	0[c]	45	2.2	62

[a] The motility data for RO8, RO8/pFlaA, OTR7, and OTR27 have been published previously and are presented here for comparison (15, 18).
[b] All results were determined from three separate experiments except for those with OTR27, which were from two separate experiments. In the case of adherence, each value is the percentage of CHSE cells with bound bacteria out of the total number of CHSE cells observed. Ten microscopic fields were counted per experiment. Double immunofluorescence labeling was used to differentially label the intracellular and extracellular bacteria. A total of at least 1,000 bacterial cells, both intracellular and extracellular, were counted for each experiment.
[c] OTR27 is motile by smooth swimming in liquid medium but, due to its nonchemotactic phenotype, cannot migrate through soft agar (18).

motY mutant was injected intraperitoneally. Moreover, the *motY* mutant complemented with the wild-type gene regained almost wild-type virulence (4×10^4 bacteria/ml) via the immersion route of infection. These results are similar to those found with mutants with partial motility (15), mutants defective in chemotaxis (18), and mutants lacking flagella (18), suggesting that motility and not the flagellin proteins is required for the invasion of rainbow trout. As a control, the *motY* mutant and the complemented strain were isolated after passage through the fish model, and the above phenotypes were confirmed to be stable.

Next, we wanted to test whether a flagellar component could serve as an adhesin and if the flagellum was needed for invasion of a fish cell line. Comparative adhesion and invasion studies with a CHSE fish cell line were done using the wild-type strain and a set of isogenic mutant strains with the following phenotypes (Table 1): nonmotile due to a paralyzed flagellum (PO2), nonmotile due to the loss of a flagellum (OTR7), partially motile due to the loss of FlaA (RO8), and nonchemotactic due to the loss of CheR (OTR27). *V. anguillarum* has previously been shown to adhere to CHSE cells (8, 22), so we chose this cell line for our analyses. Monolayers of CHSE cells were grown as previously described (10) and were infected for 2 h with 10^7 bacteria from an overnight culture of *V. anguillarum* grown in colonization factor antigen broth (1) supplemented with 2% sodium chloride. Subsequently, the bacterial cells were labeled by a previously described (4, 21) double immunofluorescence-labeling technique that differentiates intracellular and extracellular bacteria. Primary antiserum used in these studies was raised against formalin-killed whole cells of *V. anguillarum* 775-17B (16). The same infected monolayers were used for the adherence and invasion analyses.

For invasion analysis, the intracellular bacteria were counted, and the value was reported as a percentage of the total bound bacteria (Table 1). The paralyzed and partially motile mutants, PO2 and RO8, had a decreased number of intracellular bacteria compared to the wild type. When both mutations were complemented with the respective wild-type genes, the number of intracellular bacteria was approximately at the wild-type level. However, the *flhA* mutant, OTR7, which lacked flagella totally, did not show a significant decrease in the number of intracellular bacteria from that of the wild type. It is unclear why this nonmotile *flhA* mutant had a higher internalization rate than a nonmotile *motY* mutant that has a paralyzed flagellum. One explanation is that the flagellum might physically hinder the internalization of a nonmotile bacterium. Conversely, the chemotactic mutant, OTR27, was much more invasive than the wild type, suggesting that the smooth-swimming phenotype increases the ability of *V. anguillarum* to invade CHSE cells. Hyperinvasion of a chemotactic mutant has also been seen for *Salmonella enterica* serovar Typhimurium (7) and *Campylobacter jejuni* (24). To confirm that *V. anguillarum* did invade the CHSE cells, infected CHSE cells were examined by electron microscopy using standard tissue embedding techniques and immunogold labeling as previously described (15). Inside the CHSE cell, *V. anguillarum* was clearly located within a vesicle. These vesicles did not appear in uninfected CHSE cells. Taken together, these studies suggest that motility may increase the frequency of invasion by increasing the chance of collision between the pathogen and host cells.

For adherence analysis, the number of bacterium-bound CHSE cells was determined and presented as a percentage of the total number of CHSE cells counted. As shown in Table 1, *V. anguillarum* does not adhere strongly to CHSE cells. Except for a chemotactic mutant, very little difference was seen between the wild type and the mutants. The two nonmotile strains, one lacking a flagellum, OTR7, and the other containing a paralyzed flagellum, PO2, had slightly higher percentages of bound bacteria than the wild type. Since one contains a flagellum and the other does not have one at all, these differences are more likely due to the settling of bacteria onto the cell line and to their inability to swim away than to adhesins located on the flagellum. This would then increase the contact of these bacteria with the CHSE cells, increasing the likelihood of binding. On the other hand, with the nonchemotactic (*cheR*) mutant there was an increase in the number of CHSE cells with bound bacteria, as well as a slight increase in the number of bacteria bound to the CHSE cells. This mutant is a smooth swimmer and is unable to reverse (R. O'Toole and H. Wolf-Watz, unpublished results). The increase in the percentage of adherence for this bacterium is likely due to a very high rate of bacterial contact with the CHSE cells compared to that of the wild type. Therefore, the flagellum does not appear to carry an adhesin required for binding to CHSE cells.

In previous studies (8, 22), *V. anguillarum* strains were shown to bind CHSE cells at a rate that ranged from high (>100 bacteria per CHSE cell) to not at all. In comparison, our strain could be described as having a low adherence rate, 1 to 2 bacteria per CHSE cell. However, in the earlier studies using both virulent and avirulent strains (8, 22), no association between the degree of virulence of a strain and its ability to adhere in vitro to CHSE cells could be made. A possible conclusion from the present study and from the earlier studies is that adhesion may not be a prerequisite for colonization of fish by *V. anguillarum*; instead, active motility is needed. A *C. jejuni* study (11) lends support to this conclusion. Lee et al. (11)

used a bacterial-pathogen-free mouse model to analyze colonization of the intestines by *C. jejuni*. After infection, the intestines were colonized by highly motile *C. jejuni* cells. However, no evidence was found that the bacteria attached to the intestinal cells. Bacterial colonization in the absence of adherence to tissues suggests that active motility with chemotaxis may be a possible alternative to cell attachment. It is unlikely, however, that active motility and adherence are mutually exclusive, and the importance of possible adhesins cannot be ruled out.

In conclusion, by disabling the motor of the flagellum, we showed that active motility, rather than the individual components of the flagellum of *V. anguillarum*, is needed for entry into the fish host. *V. anguillarum* is an aquatic bacterium that is chemotactically motile towards fish skin and intestinal mucus (19) and that uses intestinal mucus as a nutrient source (2). Consequently, mucus should induce smooth swimming. Based on what we have seen with the CHSE cell line, smooth swimming may increase *V. anguillarum*'s probability of finding a site on the fish to invade and of passing through the mucus layers that cover both the outer surface of the fish and the intestinal tissues. Once the bacterium has invaded the fish, motility is no longer needed for the progression of vibriosis (15, 18).

Nucleotide sequence accession number. The *motY* gene sequence has been submitted to GenBank with the accession number AF176946.

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REFERENCES

1. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18:**330–337.
2. **Garcia, T., K. Otto, S. Kjelleberg, and D. R. Nelson.** 1997. Growth of *Vibrio anguillarum* in salmon intestinal mucus. Appl. Environ. Microbiol. **63:**1034–1039.
3. **Gussin, G. N., C. W. Ronson, and F. M. Ausubel.** 1986. Regulation of nitrogen fixation genes. Annu. Rev. Genet. **20:**567–591.
4. **Heesemann, J., and R. Laufs.** 1985. Double immunofluorescence microscopic technique for accurate differentiation of extracellularly and intracellularly located bacteria in cell culture. J. Clin. Microbiol. **22:**168–175.
5. **Horne, M. T., and A. Baxendale.** 1983. The adhesion of *Vibrio anguillarum* to host tissues and its role in pathogenesis. J. Fish Dis. **6:**461–471.
6. **Huang, S., and M. P. Deutscher.** 1992. Sequence and transcriptional analysis of the *Escherichia coli rnt* gene encoding RNase T. J. Biol. Chem. **267:**25609–25613.
7. **Jones, B. D., C. A. Lee, and S. Falkow.** 1992. Invasion by *Salmonella typhimurium* is affected by the direction of flagellar rotation. Infect. Immun. **60:**2475–2480.
8. **Krovacek, K., A. Faris, W. Ahne, and I. Månsson.** 1987. Adhesion of *Aeromonas hydrophila* and *Vibrio anguillarum* to fish cells and to mucus-coated glass slides. FEMS Microbiol. Lett. **42:**85–89.
9. **Kutsukake, K., Y. Ohya, and T. Iino.** 1990. Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*. J. Bacteriol. **172:**741–747.
10. **Lannon, C. N., J. R. Winton, and J. L. Fryer.** 1984. Fish cell lines: establishment and characterization of nine cell lines from salmonids. In Vitro **20:**671–676.
11. **Lee, A., J. L. O'Rourke, P. J. Barrington, and T. J. Trust.** 1986. Mucus colonization as a determinant of pathogenicity in intestinal infection by *Campylobacter jejuni*: a mouse cecal model. Infect. Immun. **51:**536–546.
12. **McCarter, L. L.** 1994. MotY, a component of the sodium-type flagellar motor. J. Bacteriol. **176:**4219–4225.
13. **McGee, K., P. Hörstedt, and D. L. Milton.** 1996. Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. J. Bacteriol. **178:**5188–5198.
14. **Milton, D. L., A. Norqvist, and H. Wolf-Watz.** 1992. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. J. Bacteriol. **174:**7235–7244.
15. **Milton, D. L., R. O'Toole, P. Hörstedt, and H. Wolf-Watz.** 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. **178:**1310–1319.
16. **Norqvist, A., and H. Wolf-Watz.** 1993. Characterization of a novel chromosomal virulence locus involved in expression of a major surface flagellar sheath antigen of the fish pathogen *Vibrio anguillarum*. Infect. Immun. **61:**2434–2444.
17. **Okunishi, I., I. Kawagishi, and M. Homma.** 1996. Cloning and characterization of *motY*, a gene coding for a component of the sodium-driven flagellar motor in *Vibrio alginolyticus*. J. Bacteriol. **178:**2409–2415.
18. **O'Toole, R., D. L. Milton, and H. Wolf-Watz.** 1996. Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum*. Mol. Microbiol. **19:**625–637.
19. **O'Toole, R., S. Lundberg, S.-Å. Fredriksson, A. Jansson, B. Nilsson, and H. Wolf-Watz.** 1999. The chemotactic response of *Vibrio anguillarum* to fish intestinal mucus is mediated by a combination of multiple mucus components. J. Bacteriol. **181:**4308–4317.
20. **Otterman, K. M., and J. F. Miller.** 1997. Roles for motility in bacterial-host interactions. Mol. Microbiol. **24:**1109–1117.
21. **Rosqvist, R., I. Bölin, and H. Wolf-Watz.** 1988. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. Infect. Immun. **56:**2139–2143.
22. **Santos, Y., I. Bandín, T. P. Nieto, J. L. Barja, A. E. Toranzo, and A. E. Ellis.** 1991. Cell-surface-associated properties of fish pathogenic bacteria. J. Aquat. Anim. Health **3:**297–301.
23. **Wang, X. H., H. L. Oon, G. W. P. Ho, W. S. F. Wong, T. M. Lim, and K. Y. Leung.** 1998. Internalization and cytotoxicity are important virulence mechanisms in vibrio-fish epithelial cell interactions. Microbiology **144:**2987–3002.
24. **Yao, R., D. H. Burr, and P. Guerry.** 1997. CheY-mediated modulation of *Campylobacter jejuni* virulence. Mol. Microbiol. **23:**1021–1031.